Effect of Human Natural Killer Cells on the Metastatic Growth of Human Melanoma Xenografts in Mice with Severe Combined Immunodeficiency

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ABSTRACT

An in vivo model for human melanoma was established with the growth of CR3 and DE5 human melanoma tumor cells following i.v. injection into C.B-17 severe combined immunodeficient mice depleted of murine natural killer (NK) cells. The ability of human NK cells to mediate antitumor activity in vivo was investigated by evaluating the number of lung nodules and survival of mice given injections of human NK cells i.v. early after injection of CR3 tumor cells. Under these conditions, human NK cells effectively reduced lung nodule counts and prolonged survival when co-injected with interleukin 2 (IL-2). Multiple injections of IL-2 given during the first 16 h post-NK injection did not further enhance the tumor reduction. Significantly increased antitumor activity against CR3 tumor cells in vivo was observed in mice receiving NK cells co-injected with IL-2 and interleukin 12 (IL-12) in comparison to NK cells and IL-2 alone. However, coinjection of IL-12 with human NK cells alone did not reduce the tumor burden. These results demonstrate the antitumor activity of human NK cells against human melanoma in severe combined immunodeficient mice and its augmentation by IL-2, alone or in combination with IL-12, suggesting that this model can be used to further investigate the interaction between human NK cells and human tumors.

INTRODUCTION

Several lines of evidence in murine tumor models have indicated that NK cells play an important role in the control of blood-borne metastases. Enhancement of NK cell activity in vivo using IFN inducers (e.g., poly I:C) has been reported to result in increased antitumor activity and prolonged survival (1); conversely, depletion of NK cells with ASGM1 antisera treatment in vivo increased metastatic growth and reduced survival time (2, 3). Beige mice, which are genetically deficient in NK cell activity, also exhibit reduced antitumor activity in vivo and in vitro (4). Furthermore, recent studies have demonstrated that increased tumor cell resistance to NK cell lysis in vitro and increased tumorigenicity in vivo correlates with increased expression of MHC Class I molecules on tumor cells (5, 6). Finally, efforts to augment the immunogenicity of tumors using the transfection of the cytokine genes, IL-2 (7) and IFN-γ (8), have also demonstrated a role for NK cells in the control of metastatic tumor growth.

It is unclear what role NK cells play in metastatic disease clinically. A positive correlation between NK cell activity and disease-free survival time has been reported for metastatic melanoma (9) and squamous cell head and neck carcinoma (10). In addition, NK cells are found in metastatic melanoma lesions (11), and their number is significantly increased following vaccine therapy (12). In vitro, melanoma cell lines are sensitive to LAK and NK cell lysis (13). Enhanced LAK activity has been associated with therapeutic responses to IL-2 alone (14) or in conjunction with IFN-α (15). However, adoptive transfer of NK cells in the form of LAK cell therapy has had little success against established metastatic disease (16). The discrepancies between the NK cell sensitivity of melanoma cells in vitro and the response rate to immunotherapy in vivo suggest that the in vitro analyses of antitumor activity may not accurately reflect the lymphocyte:tumor interaction in vivo. The development of an animal model that would allow human melanoma cells to grow and metastasize and also support the survival of functional human lymphocytes would enable the investigation of human lymphocyte:melanoma interactions with greater relevance to the clinical situation.

In this regard, SCID mice may provide a suitable model. These mice lack functional T- and B-cells due to an inability to rearrange functional T-cell receptor and immunoglobulin genes, respectively, whereas macrophages, NK cells, and other cell lineages are not affected (17). SCID mice have been found to support the growth and metastases of several human tumor cell lines as well as fresh human tumor (18—23), and the immunodeficiency of the mice has also allowed successful reconstitution with human lymphocytes with some functional capacity (24, 25). Human hematopoiesis and T-cell development using transplanted human fetal thymi and fetal liver cells demonstrated long-term reconstitution of SCID mice with circulating, functional CD4+ and CD8+ T-cells (26). The generation of both B- and T-cell responses to antigens has been demonstrated using human lymphocyte-SCID chimera (27, 28). The only report of human NK cell function in SCID mice has been an indirect observation that depletion of NK cells from donor cell populations allowed for greater clonal diversity within the repopulating human B-cell population (29).

In the present study, the ability of human NK cells to mediate antitumor activity in vivo in SCID mice bearing human melanoma metastases was investigated. Both CR3 and DE5 human melanoma cells readily metastasize in SCID mice depleted of host NK cells. Using human NK cells expanded in an in vitro culture system (30), antitumor activity against CR3 human melanoma was demonstrated, particularly when co-injected with IL-2, alone or in combination with IL-12.

MATERIALS AND METHODS

Mice. Six- to 16-wk-old male and female C.B-17 scid/scid mice, were generated from our own breeding colony using breeder pairs generously provided by George Carlson, McLaughlin Research Institute, Great Falls, MT. Mice were maintained under sterile conditions in microisolator cages and given autoclaved food and water, pH 2.5, ad libitum. Bacitracin was administered in the water weekly, prophylactically. All manipulations were carried out in a laminar flow hood. Some of the mice involved in these studies may have been exposed to pinworm infection. However, similar results were observed in mice known to be pinworm free.

Tumor Cells. CR3 and DE5 human melanomas were originally derived from a resected lymph node and s.c. metastasis, respectively, and have been previously described (21). Tumor cells were maintained in vivo in SCID mice to provide fresh tumor cells for the studies described or derived from cryopreserved tumor stocks. Briefly, s.c. tumor was resected, and a single cell suspension was prepared by mincing the tissue and forcing pieces through a
fine steel mesh. Viable cells (10^7) were then injected s.c. in 0.25 ml of PBS solution into SCID mice for serial passaging, or they were resuspended at 4 x 10^6 cells/ml of PBS for i.v. injection. Following early passages in SCID mice, a cell line was derived, CR3S, from tumor resected from the s.c. tumor site and maintained at 37°C, 5% CO_2 in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 2 mm L-glutamine (Fisher Scientific, Pittsburgh, PA), 50 I.U. of penicillin/50 µg of streptomycin (Fisher), and 5 x 10^-5 M 2-mercaptoethanol (GIBCO, Gaithersburg, MD) (complete medium).

**Cell Lines.** RPMI 8866 human B-lymphoblastoid cell line was maintained at 37°C, 8% CO_2 in RPMI 1640 supplemented with 10% FCS and 2 mm L-glutamine. K562, U937, YAC-1, and P815 cell lines were obtained from the American Type Culture Collection and maintained at 37°C, 5% CO_2 in complete medium. All cell lines were free from Mycoplasma contamination with repeated testing.

**Experimental Metastasis Model.** Mice were given injections i.p. with either ASGM1 antibody (Wako Chemical, Charlestown, NC) or NRIg (Dako Corp., Carpinteria, CA) at equal protein concentrations (125 to 250 µg/mouse) 2 days before tumor injection. To activate NK cells in vivo, polyclonal (Pharmacy LKB, Piscataway, NJ) was diluted to 1 mg/ml in PBS, and 0.1 ml was injected i.v. on Day -1. On Day 0, CR3 tumor cells were harvested from the s.c. tumor, a single cell suspension was prepared, and 10^6 viable tumor cells were injected i.v. into SCID mice. At Day 35, mice were sacrificed, and lungs were resected and fixed overnight in Bouin’s solution. Lungs were removed and placed in 70% ethanol solution, and nodules were quantitated using a dissecting microscope. For DES tumor, 10^6 viable cells from cryopreserved stock of SCID-passaged tumor cells were used for i.v. injections, and mice were sacrificed at 8 wk. Lungs with massive tumor growth that did not permit enumeration of individual nodules were assigned a value of 250. Confirmation of tumor nodules as human melanoma was obtained by H&E staining of lung sections of paraffin-embedded tissues of some samples.

**NK Cells.** Activated NK cells were generated as previously described (30). Briefly, PBMC were isolated from buffy coats of healthy individuals using Histopaque (Sigma). NK cells were purified, from short-term cultures (10 days) of PBMC with irradiated RPMI 8866 B-lymphoblastoid cells, by negative selection with indirect antitumor rosetting and density gradient centrifugation after sensitization of the lymphocytes with a cocktail of anti-CD3 (OKT3), anti-CD5 (B36.1), and anti-CD14 (BS2.1) monoclonal antibodies. Purity of cell populations was confirmed using flow cytometric analysis for expression of CD45 (GAP83.3), CD16 (3G8 or B73.1), CD56 (B159.5), CD3 (OKT3), and CD14 (BS2.1). For this, monoclonal antibodies were added to cell suspensions, incubated at 4°C for 20 min, washed, and incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G + immunoglobulin M (Fab')2 (Boehringer Mannheim Corp., Indianapolis, IN) at 4°C for 20 min. Cells were washed, fixed with 1% paraformaldehyde for 5 min, and analyzed on an EPICS Profile analyzer (Coulter Electronics, Hialeah, FL).

**Cytotoxicity Assays.** Serial dilutions of effectors were incubated with 5 x 10^5 - 3 x 10^6 111 Cr (Amersham, Arlington Heights, IL)-labeled target cells in quadruplicate wells of microtiter plates in a final volume of 200 µl at 37°C for 4 h. One hundred µl of supernatant were removed from each well, and radioactivity was quantitated in a LKB 1272 Clini gamma counter (Pharmacia). The percentage of lysis was determined using the following equation: % specific lysis = [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] x 100. Lytic units were determined using calculations as described (31). One lytic unit is the number of effector cells necessary to lyse 30% of the target cells. Transwell assays were performed in 24-well plates (Costar) using 3 x 10^5 effectors/well 6 x 10^5 or 3 x 10^5 targets/well in a final volume of 1.3 ml. SCID spleen cell suspensions were obtained using glass tissue grinders followed by lysis of RBC with Gey’s balanced salt solution containing 0.7% NH4Cl. SCID spleen cells were used at a final concentration of 3 x 10^6 cells/well. Three 100-µl aliquots were withdrawn from each well after a 4-h incubation at 37°C, and radioactivity was quantitated in a gamma counter. Spontaneous release was routinely less than 30% of maximum for human melanoma tumor cells (fresh or cultured) and less than 20% for all other cell lines. Addition of rIL-2 and/or rIL-12 to the medium did not affect spontaneous release for any tumor target.

Fluorochrome Labeling and Localization of Human NK Cells. Purified NK cell populations were resuspended at 10^6/ml in GIL diluent and added to PKH-26 (Zynaxis Cell Science, Inc., Malvern, PA), diluted 1:1000 (final concentration) in GIL diluent. The suspension was incubated for 5 min at room temperature, and the labeling reaction was stopped with the addition of FCS. Cells were washed, viability was determined using trypan blue exclusion, and cytotoxicity was measured in the standard 4-h 51Cr release assays described above. PKH-26 staining of the NK cell population was determined by two-color analysis using flow cytometry as described above. No effect on viability or in vitro cytotoxic activity was observed following staining. Mice were treated with 12.5 µl (125 µg) of ASGM1 antibody i.p. 2 days before the i.v. injection of 4 x 10^6 CR3 cells on Day 0. ASGM1 antibody (125 µg/mouse) was again administered i.p. 2 days prior to the i.v. injection of 1.8 x 2 x 10^6 PKH-26-labeled NK cells. Human rIL-2 (Biosource, Inc., Camarillo, CA) was administered i.p. to the appropriate groups. Mice were sacrificed by cervical dislocation at different time points, and lungs were resected and snap frozen in OCT medium. Snap-frozen tissue was cut at 6 µm, fixed in acetone, and coverslipped with cryoacyclon glue as per the dye manufacturer’s instructions. Slides were examined with an epifluorescent microscope with a red filter (wave length specified by manufacturer). Serial sections were cut and stained with H&E and examined using light microscopy (P. A. M.).

**Immunotherapy of Experimental Metastases.** NK cells were resuspended in PBS at appropriate concentrations and injected in a 0.25-ml final volume of PBS i.v. into ASGM1 antibody-treated mice that had received 10^6 viable CR3 tumor cells i.v. 1 h prior to NK cell injection. Except where stated, 10^6 units of rIL-2 (Biosource, Inc., Camarillo, CA) and/or 10^6 units of human rIL-12 (6 x 10^6 units/mg) (a generous gift of S. F. Wolf, Genetics Institute, Cambridge, MA) were administered i.p. immediately after NK cell injection in 0.1 to 0.2 ml of PBS. One unit of rIL-12 is defined as the amount inducing half-maximal proliferation of PHA blast cells. Lungs were harvested on Day 35 as described above for the experimental metastasis model. In established metastases experiments, mice received ASGM1 antibody injections on Day -2, 10^6 CR3 on Day 0, and a second injection of ASGM1 at 2 days before administration of NK cells and/or rIL-2.

**Statistical Analysis.** Statistical analysis of data was performed using SYSTAT (v.5.2.1 Macintosh) software. Data from separate experiments were pooled for analysis, as indicated. The F-test and Tukey’s HSD multiple ANOVA were used to analyze differences between groups based on lung nodule numbers. The F-test was used to determine that the differences between the groups were due to variance between groups and not simply to differences due to error. If the F-ratio was not significant, post hoc analysis using Turkey’s HSD multiple ANOVA was not performed. The Wilcoxon signed ranks test was used to analyze survival data.

**RESULTS**

Experimental Metastasis of Human Melanoma in SCID Mice. Because murine NK cells have previously been observed to affect the ability of human tumor cell lines to metastasize (3), the effect of SCID NK cells on the experimental metastatic growth of human melanoma cells was determined by depletion of host NK cells with ASGM1 antibody treatment (32). One day after injection of ASGM1 or NRIg control antibody i.p., polyclonal (I:C) was injected to activate NK cells via induction of IFN production (1). NK cell activity of murine splenic lymphocytes in vitro was tested using YAC-1 (NK sensitive) and P815 (NK resistant) tumor targets. Splenic cells from poly I:C + NRIg antibody-treated mice had 64.7 ± 23.2 lytic units/10^7 effectors (mean ± SD, n = 7). Lack of significant lysis of YAC-1 in the other treatment groups confirmed the efficacy of the ASGM1 treatment. P815 was not lysed by splenic cells from any group (data not shown).

In vivo, tumor growth was observed not only in the lung, but also at distant sites including brown fat, skeletal muscle, salivary gland, eye, subcutaneous sites, and visceral organs. Depletion of SCID NK cells (ASGM1 group) in vivo resulted in a significant increase in lung tumor nodules (P < 0.001) following i.v. injection of 10^6 fresh CR3 melanoma cells in comparison to mice given NRIg control antibody (Table 1). NK cell activation via polyclonal I:C injection in vivo reduced...
lung tumor nodule counts in both ASGM1 and NRIg groups by 34.1% ($P = 0.001$) and 73.3% ($P = 0.014$), respectively. Poly I:C treatment in combination with ASGM1 antibody resulted in increased antitumor activity compared to ASGM1 antibody alone, suggesting that either endogenous IFN-responsive cells other than NK cells or some residual organ-associated NK cells (33) were participating in the antitumor activity. The most extensive distant metastatic growth was observed in ASGM1 antibody-treated mice, and conversely, few if any grossly detectable metastases were found in mice receiving NRIg antibody + poly I:C.

Similarly, the injection of $10^6$ cryopreserved DES tumor cells resulted in metastatic lung tumor growth, although less in number, in most mice without NK cell depletion (Table 1). ASGM1 antibody treatment of SCID recipients significantly increased lung tumor growth observed with DES tumor cells ($P < 0.001$). A significant enhancement of lung tumor nodules following ASGM1 antibody treatment relative to NRIg antibody treatment was observed in a second experiment (data not shown). Although the lung nodule counts were very similar between groups receiving poly I:C and antibody (NRIg or ASGM1), five of six mice receiving ASGM1 antibody treatment had grossly detectable distant metastases, while the mice receiving NRIg treatment had none. As observed with CR3 tumor, the most extensive distant metastatic growth was in the ASGM1 antibody treatment groups. The lower numbers of tumor nodule growth of DE5 tumor relative to CR3 tumor are likely due, at least in part, to the use of cryopreserved cells. Lower lung tumor growth was also observed with CR3 tumor cells when using cryopreserved cells (data not shown).

ASGM1 antibody injected after establishment of metastatic growth (Day 9) did not effectively enhance CR3 lung tumor growth, and conversely, activation of NK cells following poly I:C injection (Day 11) did not reduce CR3 lung tumor growth (data not shown). The effect of ASGM1 antibody treatment is unlikely due to a direct effect on the growth of human melanoma. Flow cytometric analysis revealed that the ASGM1 antibody did not bind CR3 or DE5 melanoma cells to a significant degree (CR3, 10.5 ± 11.9%, mean ± SD, $n = 7$; DE5, 2.0 ± 3.1, $n = 5$).

These experiments confirmed the xenoreactivity of SCID NK cells against human melanoma cells during the establishment of lung metastases. Thus, all further experiments were performed in ASGM1 antibody-treated mice to eliminate this source of antitumor activity. Based on the finding that CR3 melanoma grew more readily by either s.c. or i.v. injection in SCID mice than DE5 melanoma (data not shown), CR3 melanoma cells were used in all subsequent experiments.

### Table 1. In vivo growth of human melanoma

<table>
<thead>
<tr>
<th>Mice</th>
<th>Antibody Treatment</th>
<th>poly I:C</th>
<th>Lung nodule count &lt;sup&gt;b&lt;/sup&gt; (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR3 tumor</td>
<td>18 NRIg</td>
<td>-</td>
<td>66.2 ± 27.3 (1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>18 NRIg</td>
<td>+</td>
<td>17.7 ± 10.6 (2)</td>
</tr>
<tr>
<td></td>
<td>17 ASGM1</td>
<td>-</td>
<td>179.8 ± 81.3 (3)</td>
</tr>
<tr>
<td></td>
<td>17 ASGM1</td>
<td>+</td>
<td>118.5 ± 35.1 (4)</td>
</tr>
<tr>
<td>DE5 tumor</td>
<td>6 NRIg</td>
<td>-</td>
<td>2.8 ± 2.6 (5)</td>
</tr>
<tr>
<td></td>
<td>6 NRIg</td>
<td>+</td>
<td>3.0 ± 2.1 (6)</td>
</tr>
<tr>
<td></td>
<td>6 ASGM1</td>
<td>-</td>
<td>44.8 ± 12.4 (7)</td>
</tr>
<tr>
<td></td>
<td>6 ASGM1</td>
<td>+</td>
<td>5.2 ± 2.7 (8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total number of mice per group obtained by pooling results from three separate experiments for CR3 tumor and one experiment for DE5 tumor.

<sup>b</sup> F-ratio = 39.7, $P < 0.001$ for CR3 tumor; F-ratio = 60.0, $P < 0.001$ for DE5 tumor.

<sup>c</sup> Numbers in parentheses, group. Statistical significance as determined by Tukey's HSD post hoc analysis: Groups 1 versus 3, 2 versus 4, 3 versus 5, 4 versus 6, 7 versus 8; all other relevant differences were not significant ($P > 0.05$).

**In Vitro Activity of Human NK Cells against Human CR3 Melanoma.** Using the *in vitro* culture system previously described (30), populations of human NK cells that were ≥98% CD3<sup>+</sup>, CD16<sup>+</sup>, CD56<sup>+</sup> were isolated. NK cells generated in this procedure are activated (resting lymphoblasts) and have been shown to be phenotypically, functionally, and morphologically identical to fresh NK cells. They differ only by the expression of HLA-DR, a late activation antigen, and the ability to be more readily activated. CD3+ T-cells typically accounted for 2.0 ± 2.0% (mean ± SD, $n = 17$) of the purified cell populations used in these experiments.

Fresh CR3 tumor cells were lysed effectively *in vitro* by cultured human NK cells (Fig. 1). NK cells exhibited consistently lower lytic activity against CR3 cells relative to K562 (data not shown). As expected, coinubcation of NK cells with low doses of rIL-2 (10 units/ml) and rIL-12 (1 unit/ml) enhanced cytotoxicity against fresh CR3 tumor cells (Fig. 1). Similarly, addition of rIL-2 and/or rIL-12-enhanced cytotoxicity against the CR3 cultured cell line (medium alone, 279.8 ± 150.8 lytic units/10<sup>7</sup> effectors, mean ± SD, $n = 5$; rIL-2 + rIL-12, 600.5 ± 329.2, $n = 5$; rIL-2, 503.6 ± 54.7, $n = 3$; rIL-12, 324.1 ± 97.3, $n = 3$). On the other hand, stimulation of NK cells with either a combination of IL-2 and IL-12, or rIL-12 alone, increased cytotoxicity against the CR3 tumor cell line.
HUMAN NK CELLS AND MELANOMA IN SCID MICE

Localization of Labeled Human NK Cells in CR3 Micrometastases. To determine whether adoptively transferred NK cells localize to tumor sites in the lung, cells were stained with PKH-26, a lipophilic fluorochrome that intercalates into the outer membranes of the cells. In two experiments, labeled NK cells were only detectable for up to 6 h after injection in the lung parenchyma of nontumor- and tumor-bearing SCID mice (Fig. 3). Localization of these NK cells at sites of CR3 tumor growth was observed at ≤6 h postinjection in only 3 of 10 tumor-bearing mice and not at all sites of metastatic growth. However, the clustering of NK cells observed could not be quantitated due to the staining pattern with PKH-26. Coinjection of IL-2 did not enhance survival of NK cells in the lung or localization at the site of micrometastases. Labeled NK cells were not observed in the lung parenchyma of SCID mice at later time points postinjection (examined at 18, 24, and 48 h).

Effect of Human NK Cells on Melanoma Tumor Growth in Vivo. NK cells are typically thought to interact most efficiently with hematogenous metastatic cells (2). To enhance the possibility of human NK cell interaction with the CR3 melanoma tumor cells, NK cells, with or without coinjection of IL-2, were injected i.v. 1 h after the injection of 10^6 CR3 cells. In vivo antitumor activity was determined by enumerating lung tumor nodules 35 days after the tumor challenge. Injections of rIL-2 (given at the time of NK cell injection) were used in some experimental groups to determine the IL-2 dependency of this cell population (30). To investigate the effect of prolonged exposure of IL-2 in vivo on antitumor activity of NK cells, some mice were given three i.p. injections of 10^4 units of rIL-2 8 h apart (Table 2). Although single or triple injections of rIL-2 alone had no effect on CR3 tumor growth, 5 × 10^3 NK cells with a single or triple dose of IL-2 reduced by 90% the presence of tumor nodules in the lung (P < 0.001). A significant decrease of 43% in lung tumor growth (P = 0.03) was also observed in the presence of NK cells alone.

Effect of Human NK Cells on the Survival of CR3 Tumor-Bearing Mice. ASGM1 antibody-treated mice received 5 × 10^3 NK cells, with or without rIL-2, 1 h after i.v. injection of 10^6 CR3 tumor cells, as previously described, and were studied for survival. Analysis of pooled data from two separate experiments indicated a MST of 48 days (n = 8) and 44 days (n = 6) with no treatment or rIL-2 alone, respectively (Fig. 4). Treatment with NK cells alone or with NK cells and rIL-2 increased the MST to 59.5 days (n = 6) and 63 days (n = 6) with no treatment or rIL-2 alone, respectively. Enhanced survival with the administration of either NK cells alone (P = 0.05) or with NK cells and rIL-2 (P = 0.03) was significant relative to mice given no treatment or rIL-2 alone. Multiple distant metastases were evident in all animals prior to death.

Effect of Human NK Cells on Established CR3 Melanoma Metastases. To investigate the possible interaction of human NK cells with established CR3 tumor cells in vivo, mice were treated with NK cells at various time points subsequent to tumor challenge (Table 3). As observed previously, a single injection of NK cells with rIL-2...
HUMAN NK CELLS AND MELANOMA IN SCID MICE

Reduced upon coinjection of rIL-12 with rIL-2 and NK cells relative to injection of the two cytokines alone (P < 0.001) and was 7-fold greater than that seen with the combination of rIL-2 and NK cells (P < 0.001). The dramatic reduction observed in lung tumor nodule growth is illustrated in Fig. 5. Treatment of SCID mice with NK cells, IL-2, IL-12, IL-2 + IL-12 alone, or NK cells + IL-12 had no significant effect on CR3 tumor growth.

Table 4 Modulation of the effect of NK cell injection on CR3 tumor growth by IL-12

<table>
<thead>
<tr>
<th>Mice</th>
<th>NK cells</th>
<th>IL-2</th>
<th>IL-12</th>
<th>Lung nodule count (mean ± SD)</th>
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<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>228.9 ± 24.2 (1)*</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
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<td>-</td>
<td>185.3 ± 64.9 (2)</td>
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<tr>
<td>9</td>
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<td>+</td>
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<td>233.8 ± 53.1 (3)</td>
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<tr>
<td>10</td>
<td>+</td>
<td>+</td>
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<td>93.9 ± 50.9 (4)</td>
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<td>11</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>194.5 ± 68.2 (5)</td>
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<tr>
<td>10</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>221.8 ± 41.8 (6)</td>
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<td>10</td>
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<td>202.3 ± 71.4 (7)</td>
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<td>10</td>
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Table 3 Effect of NK cells on established metastases

<table>
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<tr>
<th>Mice</th>
<th>NK cells</th>
<th>IL-2</th>
<th>Lung nodule counts (mean ± SD)</th>
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<tr>
<td>1</td>
<td>5</td>
<td>Day 0</td>
<td>178.8 ± 26.3 (1)*</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
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<td>112.0 ± 69.7 (2)</td>
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<td>147.3 ± 63.4</td>
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<td>164.3 ± 29.1</td>
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<td>3</td>
<td>3</td>
<td>Days 0, 3</td>
<td>45.7 ± 30.8</td>
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Table 4 Modulation of the effect of NK cell injection on CR3 tumor growth by IL-12

Fig. 5. Photomicrographs of representative lungs from CR3-bearing mice after adoptive transfer of human NK cells coinjected with IL-2 and IL-12. Representative lungs on Day 35 following injection of 10^6 CR3 tumor cells, A, with no treatment; B, with treatment with 10^5 units of rIL-2 and 10^5 units of rIL-12 i.p. 1 h post-CR3 injection; C, treatment with 2.5 × 10^6 NK cells i.v. + 10^5 units of rIL-2 i.p.; and D, treatment with 2.5 × 10^6 NK cells i.v. + 10^5 units of rIL-2 i.p. + 10^5 units of rIL-12 i.p.

Fig. 4. Prolonged survival of SCID mice with CR3 experimental metastases following adoptive transfer of human NK cells. Results represent pooled data from two separate experiments. Mice received 5 × 10^6 NK cells i.v. ± 10^5 units of rIL-2 i.p. 1 h postinjection of 10^6 CR3 tumor cells i.v. Statistical significance was determined using the Wilcoxon signed ranks test: no treatment versus rIL-2 alone, not significant; no treatment versus NK cells alone, P = 0.05; rIL-2 versus NK cells + rIL-2, P = 0.03; NK cells alone versus NK cells + rIL-2, not significant (P > 0.05).

Increased Effectiveness of Human NK Cells after Adoptive Transfer in the Presence of IL-2 and IL-12 in Vivo. IL-12 is a potent inducer of spontaneous cytotoxicity for NK cells (34, 35). To test whether the presence of IL-12 would enhance the activity of NK cells against melanoma growth in vivo, rIL-12 was injected alone, or in conjunction with IL-2, with NK cells (2.5 × 10^7), 1 h after 10^5 CR3 tumor challenge (Table 4). As anticipated, the lower dose of NK cells and IL-2 reduced lung tumor nodule numbers, on Day 35, to about half that observed with no treatment (P < 0.001), NK cells alone (P = 0.02), or rIL-2 alone (P < 0.001). Tumor growth was significantly administered 1 h after CR3 tumor cell injection (Day 0) reduced the tumor burden (P = 0.05). Injection of multiple doses of NK cells (Days 0 and 3, Experiment 1; Days 0, 4, and 7, Experiment 2) did not further reduce the tumor burden beyond that observed with the single dose of NK cells at Day 0. NK cells and rIL-2 injected on Day 3 (Experiment 1) subsequent to CR3 tumor cell injection reduced tumor growth somewhat, but not when injected on Days 4 or 7 (Experiment 2).

Fig. 5. Photomicrographs of representative lungs from CR3-bearing mice after adoptive transfer of human NK cells coinjected with IL-2 and IL-12. Representative lungs on Day 35 following injection of 10^6 CR3 tumor cells. A, with no treatment; B, with treatment with 10^5 units of rIL-2 and 10^5 units of rIL-12 i.p. 1 h post-CR3 injection; C, treatment with 2.5 × 10^6 NK cells i.v. + 10^5 units of rIL-2 i.p.; and D, treatment with 2.5 × 10^6 NK cells i.v. + 10^5 units of rIL-2 i.p. + 10^5 units of rIL-12 i.p.
DISCUSSION

In order to examine human lymphocyte:tumor interactions in vivo in SCID mice, we have analyzed the growth of human melanoma tumor in our experimental metastasis model which is a more efficient and quantitative system than the previously reported spontaneous metastasis model (21). In this system, it was critical to determine the potential role of endogenous murine NK cells because of previously described xenogeneic activity against human tumor targets in nude and SCID mice (3, 36, 37), and because, with the anticipated administration of human IL-2 in vivo, activation of SCID NK cells with IL-2 could interfere with human tumor growth as observed with the growth of MCF-7 breast carcinoma cells in SCID mice (38). The data presented here involving ASGM1 antibody depletion or poly I:C enhancement of SCID NK cell activity suggest that they do play a significant role in reducing experimental metastases following injection of CR3 and DE5 human melanoma cells.

Previous observations of NK cell activity against tumors in vivo would predict that NK cells would be most effective against circulating tumor cells (2). To investigate the ability of human NK cells to affect human CR3 melanoma growth in our SCID model, while tumor cells were in the circulation or the capillary beds, NK cells were injected 1 h following i.v. melanoma challenge in the presence of IL-2 and/or IL-12. The NK cells used in this study were IL-2 dependent for continued activation and enhancement (30, 39). IL-12 also potentiates the spontaneous cytotoxicity of NK cells, is mitogenic for activated NK cells, and interacts with IL-2 to enhance spontaneous cytotoxicity in an additive fashion (34, 40). NK cells were also injected several days after melanoma injection to determine the antitumor activity against established metastases.

The results indicated that activated human NK cells could have a significant antitumor effect in vivo, both in terms of tumor burden and survival, and particularly in the presence of IL-2. There was some variability in the effectiveness of NK cells and IL-2 to reduce tumor burden (Table 2 versus Table 4). We believe that this was due to either NK cell donor variability that may have allowed for valuable levels of efficacy in vivo and/or to limitations inherent in tumor nodule enumeration. Massive lung growth was assigned a value of 250, which can make the control groups artificially low, and thus, the percentage of tumor reduction. The effectiveness of the transferred human NK cells was minimal against the more established metastases, although it was still evident when cells were administered 3 days after tumor challenge (Table 3). This limited capacity of NK cells to inhibit established metastases may be due to their inability to adequately infiltrate the sites of tumor growth in the lungs or to specifically home to individual target cells. As has been suggested previously (2), the reason for their effectiveness in early metastases is that they may have a much better chance of randomly encountering circulating tumor cells in the local capillary beds of the lungs, where they are both nonspecifically trapped. Although the data reported here do not prove that the human NK cells are the direct effectors of tumor reduction, the enhanced reduction of the number of lung tumor nodules with the coinjection of IL-2 suggests that human NK cells do play a significant role in mediating the tumor reduction observed.

Several mechanisms could account for the effect of human NK cells in reducing the melanoma lung growth. First, stimulation of human NK cells by SCID xenoreagents could have induced bystander lysis of the tumor cells. Second, human NK cells could be the direct effectors of the tumor reduction. Third, IL-2 and IL-12 could stimulate NK cells to produce cytokines (e.g., IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor) (35, 41–43), some of which might induce antitumor activity from other endogenous lytic effectors (e.g., macrophages, neutrophils) without directly participating in the lysis of the melanoma cells. Finally, human NK cells could mediate the reduction by a combination of direct and indirect mechanisms. Regardless of the mechanism of CR3 tumor lysis, a major role for human NK cells is clear.

In regard to the first suggested mechanism, xenoreactivity in the form of graft-versus-host disease has been reported in SCID mice with the transfer of mature human lymphocytes (44), but this was attributed primarily to the generation of xenoreactive antibodies which are not present in our system. As murine NK cells have been implicated in xenoreactive resistance to human tumor cells (Refs. 3 and 38; Table 1), it is possible that human NK cell anti-murine activity might exist. However, tumor reduction in the presence of NK cells alone was inconsistent in our model and always substantially less than that observed with NK cells and IL-2, alone or with IL-12. Furthermore, stimulation of human NK cells with SCID spleen cells in vitro did not induce bystander lysis of CR3 tumor cells in transwell assays (data not shown). On the other hand, NK cells alone were effective in prolonging survival of melanoma-bearing mice (Fig. 3). However, since distant metastases were observed in all mice in the weeks immediately prior to death, it is possible that lung tumor growth was not the direct cause of mortality, accounting for the similar survival curves in mice given injections of NK cells alone or NK cells with IL-2. Thus, overall, it does not seem likely that xenoreactivity of human NK cells against SCID tissue could account for the tumor reduction observed in the lung.

Analysis of fluorochrome-labeled NK cells indicated that NK cells are present in the lungs of both nontumor- and tumor-bearing SCID mice at 6 h (Fig. 3), but absent at 18 h, and do not appear to localize to sites of tumor growth, suggesting that, if NK cells mediate tumor reduction directly, it has to occur within the initial 18 h postinjection when both tumor cells and NK cells are in the circulation or capillary beds. The apparent requirement for IL-2 for the antitumor activity in vivo also supports the notion of an interaction of human NK cells and CR3 tumor cells occurring within the first few hours postinjection. Human IL-2 has a half-life of approximately 4 min in mice, and thus, one injection of 10⁴ units of IL-2 would be expected to result in detectable serum titers for no more than 2 h (45). The IL-2 enhancement of NK cell antitumor activity in melanoma-bearing SCID mice may be a function of the IL-2 dependency of the in vitro expanded NK cells used in this system (30). Proliferation of human NK cells, however, does not appear to be a requirement for activity based on several observations. (a) The short half-life of human IL-2 in mice and the presence of NK cells in SCID lung parenchyma for only a short time make it unlikely that significant proliferation occurs within this window of time. (b) IL-12 alone is mitogenic for activated NK cells in vitro (42), but NK cells were ineffective against CR3 melanoma cells in vivo when coinjected with IL-12 (Table 4). (c) IL-12 inhibits the IL-2-induced proliferation of activated human NK cells (42), but the antitumor activity of NK cells was significantly enhanced when both IL-2 and IL-12 were coinjected (Table 4).

The potentiation of NK cell activity with low doses of IL-12 in vivo in combination with IL-2 is noteworthy, particularly in light of the lack of NK cell antitumor activity in the presence of IL-12 alone (Table 4). This finding suggests that spontaneous cytotoxicity generated by IL-12 in vitro against tumor cell lines may not be indicative of its functionality in vivo against fresh tumor cells (34, 40). The lack of effect of IL-12 when injected alone may also have been a function of the low doses of IL-12 administered, but it is unlikely. Although the half-life of human IL-12 in mice is not known, the half-life of murine IL-12 in vivo is 24 to 28 h.⁴ Although larger doses of human IL-12 may enhance human NK cell antitumor activity, it seems likely that the efficacy of the low dose of IL-12 administered in these experiments in stimulating human NK cells in vivo was at least compa-
rable to that of the high doses of IL-2 administered, due to the differences in half-life. In any case, the low dose of IL-12 did have a significant effect on antitumor activity when combined with NK cells and IL-2. This finding suggests that IL-12 may allow IL-2 to mediate its effect in vivo at much lower doses and, thus, may circumvent the toxicity of high doses of IL-2 clinically. IL-12 is also unique in its potentiation of cytoxicity of both NK cells and T-cells; thus, IL-12 may prove to be a very potent immunotherapeutic agent in the treatment of tumors.

NK cell lysis of CR3 tumor cells in vitro indicated that direct cell-to-cell interaction is necessary (Fig. 2) and that lytic potential could be enhanced by low doses of IL-2 and/or IL-12 (Fig. 1). IL-2 and IL-12 have been found to stimulate NK cell production of IFN-γ and TNF-α (42, 46, 53). Cultured human NK cells did not produce detectable IFN-γ or TNF-α in response to IL-2 tumor cells alone in vitro, but did in response to IL-2 and/or IL-12 with 20 to 40 h of stimulation. Production of human NK cell-derived TNF-α in vivo could stimulate responsive murine antitumor effectors, whereas IFN-γ is species specific (46, 47). However, CR3 tumor cells were insensitive to growth inhibition by recombinant TNF-α and IFN-γ in vitro at doses as high as 500 units/ml, making it unlikely that these cytokines could have a direct antitumor effect in vivo. It, therefore, seems likely that a direct CR3 tumor cell:melanoma tumor interaction is occurring in vivo in the capillary beds of the lung parenchyma.

The antitumor activity of human T-cells and LAK cells in SCID mice has been recently described using established human tumor cell lines. Significant prolongation of survival of SCID mice given injections of Daudi lymphoma cells was observed following adoptive transfer of tumor-specific T-cells (48). Administration of anti-CD3-activated T-cells from normal donors also prolonged survival of SCID mice challenged with the HT-29 human colon carcinoma cell line (49). In addition, human LAK cells preincubated with antibody specific for a colon-associated antigen and polyethylene glycol effectively reduced established tumor burden and enhanced survival (50). However, human LAK cells without antibody were ineffective, suggesting that the tumor-specific antibody was necessary for LAK cell targeting to human tumor cells in vivo. NK cells in an in vivo situation and, perhaps, the elucidation of mechanisms of NK resistance by established tumor metastases. Genetic manipulation of the tumor to produce or to inhibit production of a particular cytokine and its effect on antitumor activity can also be readily addressed in such a system. Clarification of the most effective mechanisms of tumor lysis by NK cells could make NK cells a viable immunotherapeutic strategy to eliminate tumor cells that evade T-cell elimination by reducing or eliminating MHC antigen expression.

Addendum

During the review of the manuscript, human T-cells in the presence of IL-7 (51) or IL-2 (52) were demonstrated to effectively mediate reduction of human colon carcinoma and melanoma, respectively, in SCID mice.

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769

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HUMAN NK CELLS AND MELANOMA IN SCID MICE


Effect of Human Natural Killer Cells on the Metastatic Growth of Human Melanoma Xenografts in Mice with Severe Combined Immunodeficiency

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